In addition, the disease control rate was 100 %, showing a sufficient chemotherapeutic effect in patients with recurrent disease. The hematological toxicities observed were mild and treatable. Although grade 3 or 4 anemia occurred in 1 patient each, no platelet transfusion or G-CSF was required. As for non-hematological toxicity, sensory nerve disorder of grade 1, which is peculiar to oxaliplatin therapy, developed in all 7 patients, but did not require discontinuation of therapy.

Although the initiation of therapy was delayed by 1 week in 13 (44.8 %) cycles, there was only 1 cycle in which the reason for the delay was failure to satisfy the requirements for initiation of the next cycle of therapy; specifically, the reason was an unrestored neutrophil count. In this study, the S-1 dose was set at 80–120 mg/body/day, and the oxaliplatin dose at 100 mg/m^2. S-1 at a dose of 80 mg/m^2 and oxaliplatin at a dose of 130 mg/m^2 are reported to be common in SOX therapy for colorectal carcinoma. In these previous reports, S-1 or oxaliplatin therapy was completed without dose reductions in 57 and 81 %, respectively, showing rather low rates of complete treatment [9, 16]. In our study, PD occurred in 3 of the 7 patients, requiring discontinuation of therapy. However, none of the criteria for reduction of the oxaliplatin dose were met in any of the patients, and the rate of complete treatment was 100 %. SOX therapy using an oxaliplatin dose of 100 mg/m^2 appears to promote good compliance. The time required for intravenous administration of oxaliplatin on day 1 was 135 min including premedication, allowing outpatient management of patients. Thus, the patients maintained their QOL while being treated.

The results of this pilot study suggest that the SOX regimen is an effective antitumor therapy for recurrent adenocarcinoma of the uterine cervix, and is associated with minimal adverse effects. This therapy deserves further investigation in a phase-II clinical study to examine its potential as a second-line therapy for recurrent adenocarcinoma of the uterine cervix, a malignancy that is difficult to treat in daily clinical practice. The present pilot study has provided important findings that may contribute to improving the prognosis for patients with adenocarcinoma of the uterine cervix.

Conflict of interest None of the authors of this manuscript has any conflicts of interest to declare.

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Meta-analysis of epoetin beta and darbepoetin alfa treatment for chemotherapy-induced anemia and mortality: Individual patient data from Japanese randomized, placebo-controlled trials

Yasuo Ohashi,[1,9] Yukari Uemura,[1] Yasuhito Fujisaka,[2,10] Toru Sugiyama,[3] Hironobu Ohmatsu,[4] Noriyuki Katsumata,[5,11] Rumiko Okamoto,[6] Nagahiro Saijo[7,12] and Tomomitsu Hotta[8,13]

[1]Department of Biostatistics, School of Public Health, University of Tokyo, Tokyo; [2]Respiratory Medicine, Osaka Medical College Hospital, Takatsuki; [3]Department of Obstetrics and Gynecology, Iwate Medical University School of Medicine, Morioka; [4]Division of Thoracic Oncology, National Cancer Center Hospital East, Kashiwa; [5]Department of Medical Oncology, National Cancer Center Hospital, Tokyo; [6]Department of Chemotherapy, Tokyo Metropolitan Cancer and Infectious Diseases Center Komagome Hospital, Tokyo; [7]Department of Medical Oncology, Kinki University Faculty of Medicine, Osakasayama; [8]Department of Internal Medicine, National Hospital Organization, Nagoya Medical Center, Nagoya, Japan

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Erythropoiesis-stimulating agents (ESA) reduce the need for transfusions and improve the quality of life in patients receiving chemotherapy, but several clinical trials have suggested that ESA might have a negative impact on survival. To evaluate the efficacy and safety of ESA, epoetin beta and darbepoetin alfa, including their impact on overall survival and thromboembolic events, we conducted an individual data-based meta-analysis of three randomized, placebo-controlled trials studying Japanese patients with chemotherapy-induced anemia. All trials were conducted in compliance with Good Clinical Practice. A total of 511 patients with solid tumor or lymphoma (epoetin beta or darbepoetin alfa, *n* = 273; placebo, *n* = 238) were included. The ESA significantly reduced the risk of transfusion (relative risk, 0.47; 95% confidence interval, 0.29–0.76). No significant effect of the ESA on overall survival was observed (unadjusted hazard ratio, 1.00; 95% confidence interval, 0.75–1.34). A prespecified subgroup analysis showed no strong interaction between the baseline hemoglobin concentration and the effect of ESA on overall survival. Among the ESA-treated patients, the highest hemoglobin achieved during the treatment period in each patient had no impact on mortality. No increase in thromboembolic events was observed in the ESA-treated patients (0.7% *vs* 1.7% placebo). The ESA reduced the risk of transfusion without a negative impact on the survival of patients with chemotherapy-induced anemia. (*Cancer Sci* 2013; 104: 481–485)

A number of studies have reported that erythropoiesis-stimulating agents (ESA) are effective in improving anemia and that ESA decrease red blood cell (RBC) transfusion needs and improve the quality of life in cancer patients with chemotherapy-induced anemia (CIA).[1–3]

As an ESA, epoetin alfa was approved in the United States of America (USA) for CIA in 1993 and in Europe, epoetin alfa and epoetin beta were approved in 1994. Subsequently, darbepoetin alfa received European Union (EU) approval in 2001 and USA approval in 2002. Since then, ESA have been commonly used in clinical practice. However, in a clinical trial of epoetin alfa administered to chemotherapy-treated patients with metastatic breast cancer[4] and a clinical trial of radiation-treated head and neck carcinoma patients,[5] reduced survival results of ESA groups compared with placebo groups were reported. After these reports, similar findings from multiple trials were published,[6–9] suggesting negative effects of ESA on cancer patients' prognosis.

In light of these reports, an Oncologic Drugs Advisory Committee met in 2004, 2007 and 2008 in the USA to discuss and investigate the risks of ESA as a CIA treatment. As a result, a boxed warning was added to the product label of each ESA describing the risk of ESA worsening life prognosis and these warnings have remained, with multiple revisions.

Preclinical studies suggested that tumor cells express erythropoietin receptors and that ESA activate these receptors to induce or promote tumor growth.[10,11] However, technical issues (such as anti-erythropoietin receptor antibody's lack of specificity for erythropoietin receptor) have limited the validity of the findings.[12,13]

Several meta-analyses of randomized controlled trials exploring the risk of worsening the life prognosis for cancer patients on ESA have been reported.[14–19] However, the majority of the clinical trials reviewed in these meta-analyses were conducted with hemoglobin (Hb) concentrations higher than the standard stated in the current package inserts of ESA in the USA and the EU, that is, the Hb concentrations at the beginning of ESA therapy to be 10 g/dL or lower, and the target Hb concentration of 10–12 g/dL or the minimum level to avoid RBC transfusion. Moreover, some clinical trials were conducted with patients for whom treatments other than ESA were indicated (i.e. patients not receiving chemotherapy).

Erythropoiesis-stimulating agents have been marketed in Japan with indications for renal anemia, autologous blood collection and anemia of prematurity; however, their use for CIA is not yet approved. The present study analyzed three placebo-controlled trials conducted from 2006 to 2009 as developmental clinical trials in compliance with Good Clinical Practice (GCP),[20–22] to investigate the possible effects of ESA epoetin beta and darbepoetin alfa on survival using individual patient data provided by Chugai Pharmaceutical Co., Ltd, and Kyowa Hakko Kirin Co., Ltd.

Materials and Methods

Study selection and data collection. Five randomized placebo-controlled trials investigating epoetin beta or darbepoetin alfa were conducted in compliance with GCP. The trials enrolled

[9]To whom correspondence should be addressed.
E-mail: ohashi@epistat.m.u-tokyo.ac.jp
[10]Present address: Department of Medical Oncology, Kinki University Faculty of Medicine, Osakasayama, Japan.
[11]Present address: Department of Medical Oncology, Nippon Medical School Musashikosugi Hospital, Kawasaki, Japan.
[12]Present address: Japanese Society of Medical Oncology, Tokyo, Japan.
[13]Present address: National Cancer Center, Tokyo, Japan.

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Japanese patients who had solid tumors or lymphomas and CIA. Three of the five trials were selected for this meta-analysis. (Two studies on epoetin beta[23,24] were excluded from the analysis because the survival period was not designated as one of the study end-points in the protocols prior to the start of the studies.)

Details of the three individual trials are summarized in Table 1. The types of cancer were limited to patients with lung or gynecological tumors receiving platinum-containing chemotherapy in the Fujisaka trial of epoetin beta[22] and the Katakami trial of darbepoetin alfa.[20] The dose-finding study of darbepoetin, that is, the Suzuki trial,[21] enrolled patients with any type of solid tumor or malignant lymphoma receiving chemotherapy (not limited to platinum-containing chemotherapy). In the epoetin beta clinical trial,[22] inclusion criteria of the Hb concentration was between 8.0 and 10.0 g/dL, whereas in the two darbepoetin alfa clinical trials,[20,21] the Hb concentration standard at the time of study enrollment was initially 11.0 g/dL or lower. However, the Hb standard was changed to 10.0 g/dL or lower during the Suzuki trial.[21] The standard for discontinuation of epoetin beta administration in the Fujisaka trial was a Hb concentration exceeding 12.0 g/dL. In the two darbepoetin alfa trials, the discontinuation standard was initially a Hb concentration exceeding 13.0 g/dL; however, this standard was amended during both trials to a Hb concentration exceeding 12.0 g/dL.

Individual patient data were provided to the University of Tokyo, Tokyo, Japan, from Chugai Pharmaceutical Co., Ltd, Tokyo, Japan, and Kyowa Hakko Kirin Co., Ltd., Tokyo, Japan in a standardized format. All data were anonymized.

End-points. The efficacy end-points of the meta-analysis were RBC transfusion and transfusion trigger during the period from week 5 of the ESA treatment to the end of the treatment period. The transfusion trigger is defined as either receiving RBC transfusion or a Hb concentration below 8 g/dL. The maximum length of the ESA administration period was 12 weeks. The two end-points for safety were overall survival starting from the beginning of ESA administration and thromboembolic events (TEE). The TEE include cerebral infarction, cardiac infarction, pulmonary embolism, venous thrombosis, deep-vein thrombosis, superior vena cava obstruction, arteriosclerosis obliterans and thrombophlebitis.

Statistical analysis. The analytical software SAS (version 9; SAS Institute Inc., Cary, NC, USA) was used for all analyses, using a full analysis set. Individual patient data were pooled without stratification for study. For the efficacy end-points of RBC transfusion and transfusion trigger, Kaplan–Meier estimates of the cumulative incidence of each parameter were calculated in the meta-analysis and a *Z*-test was conducted for comparison between groups using the standard error based on Greenwood's formula. Moreover, for the rate of end-points that occurred during the trials, the relative risk (RR) and its 95% confidence interval (CI) were determined.

For overall survival, we drew a Kaplan–Meier plot for all patients without stratification and for each subgroup based on background characteristics such as the trial, cancer type, Hb concentration category at baseline and Eastern Cooperative Oncology Group performance status. A Cox regression model was used to determine the hazard ratio (HR) of the ESA-treated group to the placebo-treated group and for interactions between the effect of ESA and background factors. The tied data were processed using the exact method.[25]

We also conducted a landmark analysis at 3 months for the ESA-treated group and analyzed the average Hb concentration and highest Hb concentration achieved during the 3-month treatment period using a Cox regression model. Patients who responded well to ESA treatments, with high levels of average Hb concentrations during this 3-month period, might be the patients with better prognoses; therefore, clinically important factors such as baseline age, primary disease or metastatic disease were included in the analyses as adjustment factors.

Results

Description of trials. Our meta-analysis examined three randomized, placebo-controlled trials with a total of 511 CIA patients with lung cancer ($n = 258$), gynecological cancer ($n = 138$), malignant lymphoma ($n = 60$) or other types of cancer ($n = 55$) such as breast cancer, who were administered epoetin beta or darbepoetin alfa ($n = 273$) or placebo ($n = 238$). The imbalances in the numbers of patients assigned to the ESA-treated group and the placebo-treated group and in the proportions of cancer types between these two groups were caused by the Suzuki[21] trial, which included two arms of darbepoetin alfa. Details of the patients included in the meta-analysis are summarized in Table 2. Except for type of cancer, the baseline characteristics of the patients were generally well balanced between the ESA-treated group and the placebo-treated group.

Efficacy. The percentage of patients who received RBC transfusions from week 5 until week 14 were 9% in the ESA-treated group and 21% in the placebo-treated group ($P < 0.001$; Fig. S1) and the risk of transfusion was reduced in the ESA-treated group by 53% (relative risk [RR], 0.47; 95% CI, 0.29–0.76). The risk of transfusion for major cancer types was RR 0.35 (95% CI, 0.17–0.73) for lung cancer and RR 0.26 (95% CI, 0.08–0.87) for gynecological cancer. The risk of transfusion trigger from week 5 was RR 0.50 (95% CI, 0.38–0.67; Fig. S2).

Overall survival. The median follow-up period for overall survival of all patients was 13.3 months (min.–max., 1.1–19.4 months). The unadjusted HR for overall survival of all

Table 1. Details of the three clinical trials examined in the meta-analysis

Study	*n* (Total, 511)	Drug	Dosage	Treatment duration	Hemoglobin at enrollment	Hemoglobin ceiling	Cancer	Chemotherapy
Katakami *et al.* 2008[20]	207	Darbepoetin alfa	2.25 mcg/kg qw s.c.	Max. 12 weeks	≤11.0 g/dL	13.0 g/dL 12.0 g/dL†	Lung, gynecological	Platinum containing
Suzuki *et al.* 2008[21]	123	Darbepoetin alfa	4.50 mcg/kg 6.75 mcg/kg q3w s.c.	Max. 12 weeks	≤11.0 g/dL ≤10.0 g/dL†	13.0 g/dL 12.0 g/dL†	Solid, malignant lymphoma	Any
Fujisaka *et al.* 2011[22]	181	Epoetin beta	36 000 IU qw s.c.	Max. 12 weeks	8.0–10.0 g/dL	12.0 g/dL	Lung, gynecological	Platinum containing

†After amendment of protocol. qw, every week; q3w, every 3 weeks; s.c., subcutaneous injection.

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Table 2. Baseline characteristics of patients with chemotherapy-induced anemia

Background factor	ESA (n = 273)	Placebo (n = 238)
Sex (%)		
Male	121 (44.3)	102 (42.9)
Female	152 (55.7)	136 (57.1)
Age (years) (%)		
<65	155 (56.8)	148 (62.2)
≥65	118 (43.2)	90 (37.8)
Bodyweight† (kg)	53.8 ± 9.5	53.6 ± 9.4
Type of cancer (%)		
Lung cancer	130 (47.6)	128 (53.8)
Gynecological cancer	68 (24.9)	70 (29.4)
Malignant lymphoma	40 (14.7)	20 (8.4)
Other	35 (12.8)	20 (8.4)
Tumor diagnosis (%)		
Primary	184 (67.4)	173 (72.7)
Recurrent	89 (32.6)	65 (27.3)
ECOG performance status (%)		
0	143 (52.4)	115 (48.3)
1	120 (44.0)	115 (48.3)
2	10 (3.7)	8 (3.4)
Hemoglobin (g/dL) (%)		
≤9	84 (30.8)	71 (29.8)
>9–10	101 (37.0)	98 (41.2)
>10	88 (32.2)	69 (29.0)
Endogenous EPO† (mU/mL)	86.4 ± 151.5	85.1 ± 83.3

†Mean ± SD. ECOG, Eastern Cooperative Oncology Group; EPO, erythropoietin; ESA, erythropoiesis-stimulating agents; SD, standard deviation.

patients was 1.00 (95% CI, 0.75–1.34) and no influence of ESA was identified (Fig. 1). The mortality results for all patients and stratified by trials and baseline characteristics are shown in Figure 2. None of the variables (including cancer type and Hb concentration) influenced mortality. The majority of deaths were cancer-related in both the ESA-treated group 95% (94 of 99) and the placebo-treated group 97% (85 of 88).

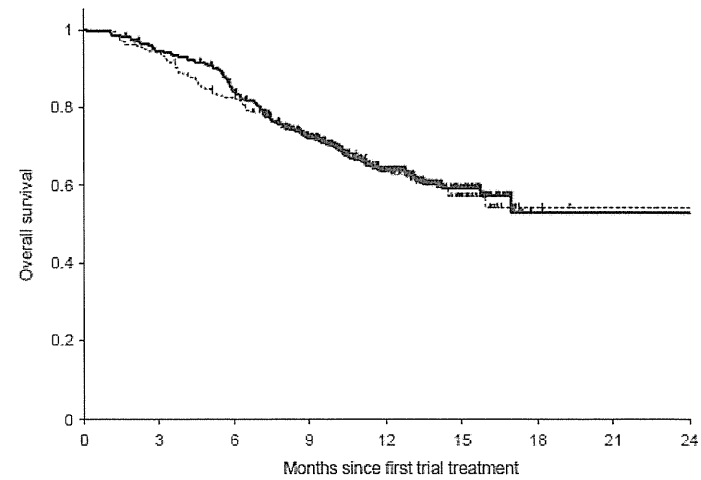
The mortality rate (i.e. number of deaths) during the treatment period (the active study period) was seven patients in the ESA-treated group and seven patients in the placebo-treated group.

The landmark analysis at 3 months for the ESA-treated patients (n = 256) indicated a tendency for patients with a mean Hb concentration of 11–11.5 g/dL during the 3-month treatment period (n = 39) to have the lowest risk of mortality (adjusted HR, 0.43; 95% CI, 0.17–1.07; reference, mean hemoglobin <10 g/dL). However, the highest Hb concentration achieved during the ESA-treatment period in each patient (mean, 11.7 g/dL; min.–max., 7.7–15.4 g/dL) had no impact on mortality.

Thromboembolic events. The frequency of TEE was 0.7% in the ESA-treated patients and 1.7% in the placebo-treated patients. The TEE reported in the ESA group were one case each of axillary vein thrombosis and pulmonary embolism and in the placebo group, one case each of cerebral infarction, hemorrhagic cerebral infarction, superior vena cava obstruction and arteriosclerosis obliterans. There was no death due to TEE.

Discussion

Our meta-analysis based on individual patient data with a median follow-up period of over 1 year did not identify an increased risk of mortality due to the ESA, epoetin beta and darbepoetin alfa. A meta-analysis published by the Cochrane Collaboration, similarly based on the individual patient data of all 13 933 patients (including those other than CIA) from 53 studies, reported an increased mortality risk of 17% (HR, 1.17; 95% CI, 1.06–1.30) by ESA administration based on overall survival during the active study period.[14] However, that meta-analysis found that when the analysis was limited to the cases of 10 441 chemotherapy-treated patients from 38 studies, an increase in the mortality risk was 10% (HR, 1.10; 95% CI, 0.98–1.24).[14]



	Hazard ratio (95% CI)	1-year survival rate	
		ESA	Placebo
All patients	1.00 (0.75 – 1.34)	0.64	0.64

Fig. 1. Overall survival. Kaplan–Meier curve for all patients treated with erythropoiesis-stimulating agents (ESA) or placebo. The median follow-up period for overall survival of all patients was 13.3 months (min.–max., 1.1–19.4 months). The unadjusted hazard ratio for overall survival of all patients was 1.00 (95% confidence interval [CI], 0.75–1.34) with a 1-year survival rate of 64% for the ESA-treated group and placebo-treated group.

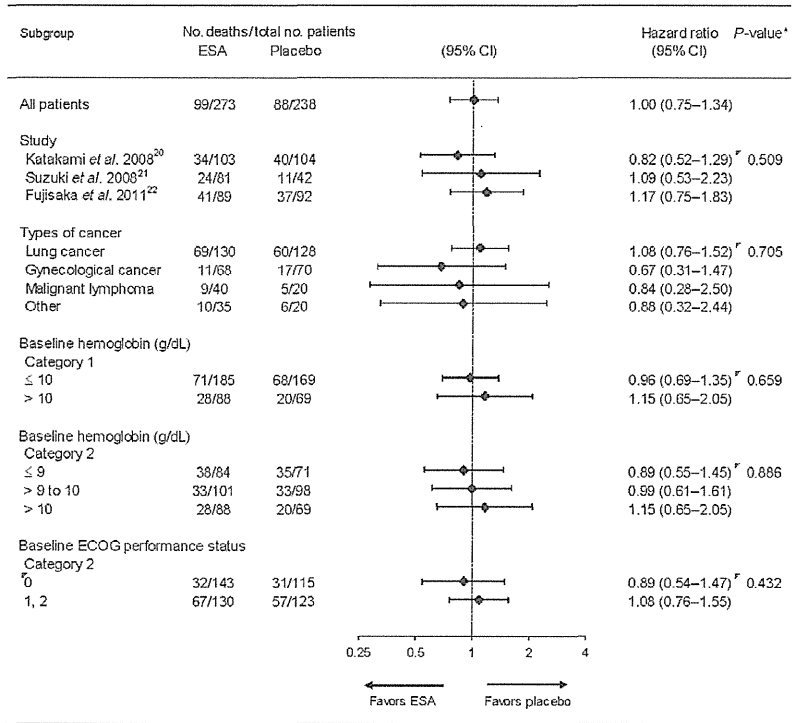


Fig. 2. Mortality risk stratified by trials and patient characteristics. Forest plots of the hazard ratio (HR) for overall survival from the three trials for all patients and stratified by trial and baseline characteristics. The position of each diamond indicates the HR estimate. Horizontal lines indicate the 95% confidence interval (CI). None of the variables (including cancer type and hemoglobin concentration) influenced patient mortality. *Test for interaction. ECOG, Eastern Cooperative Oncology Group.

There are several differences between our analysis and that of the Cochrane Collaboration. First, our analysis included patients with CIA only, which is the approved indication for ESA treatment in the EU and USA. Second, our analysis examined randomized, placebo-controlled trials conducted in compliance with GCP as developmental clinical trials by pharmaceutical companies between 2006 and 2009, after discussion of the possible contribution of ESA to decreased life prognosis. Therefore, patient selection and ESA administration in the three trials were conducted under close supervision and observation by physicians throughout the study period. The third difference is that all three trials analyzed the measured Hb concentration at least once a week during the study period and when the Hb concentration exceeded the set standard level the ESA administration was discontinued immediately, as indicated by the protocol. Thus, the increased risk associated with ESA was not observed in our analysis of Japanese CIA patients who were appropriately selected, underwent close Hb concentration monitoring and received strict dosing adjustment of ESA.

The analysis for each background factor showed that no variables (including baseline Hb concentration) influenced mortality. Thus, we conducted a landmark analysis starting from the 3-month time point for the relationship between Hb concentration after ESA administration and mortality (the subjects for the analysis were the patients with a follow up of 3 months or more). This analysis revealed that the highest Hb levels achieved during the ESA-treatment period for each patient were within the range of 7.7–15.4 g/dL and the Hb level did not influence mortality. This finding indicates that a temporary rise in Hb concentration to a high level does not negatively affect mortality.

Moreover, as an exploratory investigation, we used a model for predicting the patients whose Hb concentrations respond well to ESA administration using the patients' background factors. We defined good responders as those with a >0.5 g/dL increase in Hb concentration when the average first-month Hb was compared with the baseline. With this model, we extracted the patients who were expected to be good responders from the placebo-treated group and matched them with ESA-treated patients with good responses. This matching was implemented with randomly sorted ESA-treated patients and the HR was estimated after one-to-one matching. For the sensitivity analysis, the above analysis was iterated 10 times and the range of estimated HR was 0.54–0.83 (standard error [SE], 0.30–0.32) for the good responders and 0.82–1.10 (SE, 0.19–0.20) for the remainder (the non-good responders). If the response to ESA affects mortality and overall survival is improved in the good responders, the HR would be <1; conversely, if the ESA have a negative influence on the non-good responders, the HR would be over 1. However, we found no significant difference between these two subgroups and therefore the results suggest that the level of Hb concentration response to ESA does not change the degree of influence of ESA on mortality.

In the present study, no increase in TEE was observed in the ESA-treated patients (0.7% *vs* 1.7% placebo). Due to the low incidence of TEE and the lack of a structured approach for identifying TEE we could not conduct a detailed analysis, but in general the incidence of TEE in Japanese patients is considered to be low.[26] Previous studies of ESA (not included in the present study) showed that few Japanese CIA patients treated with ESA or placebo experienced TEE.[23,24,27,28]

As in previous reports,[17,18] the present analysis further confirms that ESA reduce the need for RBC transfusions (RR, 0.47). However, none of the trials in the present study showed any significant difference in quality of life between the ESA-treated and placebo-treated groups. Although a higher percentage of patients received RBC transfusions in the placebo-treated group than in the ESA-treated group, the mean changes in the Functional Assessment of Cancer Therapy-Anemia total fatigue subscale score were better in the ESA-treated group.

In conclusion, treatment with the ESA epoetin beta and darbepoetin alfa reduced the risk of transfusion without a negative impact on overall survival in Japanese patients with CIA where appropriate patient selection, monitoring of Hb concentration and strict dosing adjustment were conducted. Further investigations regarding Hb concentrations after ESA adminis-

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tration are needed, as is a cohort study on the relationship between the response to ESA administration and mortality.

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Disclosure Statement

Y.O. has received honoraria from and has had a consultant or advisory relationship with Chugai Pharmaceutical Co., Ltd and Kyowa Hakko Kirin Co., Ltd. In addition, Y.O. is a Chairman of the Board in Statcom Co., Ltd and has owned stocks of Statcom Co., Ltd. T.S. received honoraria and research funding from Chugai Pharmaceutical Co., Ltd. N.K. received honoraria from Chugai Pharmaceutical Co., Ltd and Kyowa Hakko Kirin Co., Ltd. N.S. received honoraria from Chugai Pharmaceutical Co., Ltd and Kyowa Hakko Kirin Co., Ltd. In addition, N.S. received research funding from Chugai Pharmaceutical Co., Ltd. T.H. had an advisory relationship with Chugai Pharmaceutical Co., Ltd and Kyowa Hakko Kirin Co., Ltd (the advisory role was defined in the delegated research contract between National Hospital Organization, Nagoya Medical Center and each company). In addition, T.H. received research funding from Chugai Pharmaceutical Co., Ltd. All remaining authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Kaplan–Meier curve of the time to first red blood cell transfusion during the period from week 5 of treatment to the end of the treatment period for all patients treated with erythropoiesis-stimulating agents or placebo.

Fig. S2. Kaplan–Meier curve of the time to transfusion trigger during the period from week 5 of treatment to the end of the treatment period for all patients treated with erythropoiesis-stimulating agents or placebo.

Checkpoint Kinase Inhibitor AZD7762 Overcomes Cisplatin Resistance in Clear Cell Carcinoma of the Ovary

Hiroaki Itamochi, MD, PhD, Mayumi Nishimura, PhD,† Nao Oumi, PhD,† Misaki Kato, PhD,† Tetsuro Oishi, MD, PhD,* Muneaki Shimada, MD, PhD,* Shinya Sato, MD, PhD,* Jun Naniwa, MD, PhD,* Seiya Sato, MD, PhD,* Akiko Kudoh, MD,* Junzo Kigawa, MD, PhD,† and Tasuku Harada, MD, PhD**

Objective: Checkpoint kinase (Chk) inhibitors are thought to increase the cytotoxic effects of DNA-damaging agents and are undergoing clinical trials. The present study was aimed to assess the potential to use the Chk1 and Chk2 inhibitor, AZD7762, with other anticancer agents in chemotherapy to treat ovarian clear cell carcinoma.

Methods: Four ovarian clear cell carcinoma cell lines were used in this study. We treated the cells with AZD7762 and anticancer agents, then assessed cell viability, cell cycle distribution, apoptosis, and the expression of protein in apoptotic pathways and molecules downstream of the Chk signaling pathways. We also investigated the effects of these drug combinations on tumor growth in a nude mouse xenograft model.

Results: Synergistic effects from the combination of AZD7762 and cisplatin were observed in all 4 cell lines. However, we observed additive effects when AZD7762 was combined with paclitaxel on all cell lines tested. AZD7762 effectively suppressed the Chk signaling pathways activated by cisplatin, dramatically enhanced expression of phosphorylated H2A.X, cleaved caspase 9 and PARP, decreased the proportion of cells in the gap 0/ gap 1 phase and the synthesis-phase fraction, and increased apoptotic cells. Combinations of small interfering RNA against Chk 1 and small interfering RNA against Chk2 enhanced the cytotoxic effect of cisplatin in both RMG-I and KK cells. Finally, treating mice-bearing RMG-I with AZD7762 and cisplatin significantly suppressed growth of tumors in a xenograft model.

Conclusions: The present study indicates that chemotherapy with AZD7762 and cisplatin should be explored as a treatment modality for women with ovarian clear cell carcinoma.

Key Words: Clear cell, Cisplatin, Resistance, Ovarian carcinoma, Checkpoint kinase

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Clear cell carcinoma of the ovary is recognized in the World Health Organization classification of ovarian tumors as a distinct histologic entity, and its clinical behavior is distinctly different from other epithelial ovarian cancers.[1] Clear cell carcinoma accounts for approximately 4% to 12% of epithelial ovarian cancers in the United States and, for unknown

*Department of Obstetrics and Gynecology, Tottori University School of Medicine, and †Tottori University Hospital Cancer Center, Yonago, Japan.

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Address correspondence and reprint requests to Hiroaki Itamochi, MD, PhD, Department of Obstetrics and Gynecology, Tottori University School of Medicine, 36-1 Nishicho, Yonago 683-8504, Japan. E-mail: itamochi@med.tottori-u.ac.jp.

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reasons, more than 20% of such cancers in Japan. The poor prognosis of patients with advanced disease may reflect the resistance of clear cell carcinoma to conventional platinum-based chemotherapy.[2,3]

Several mechanisms involved in drug resistance have been proposed, including decreased drug accumulation, increased drug detoxification, increased DNA repair activity, and up-regulation of growth factor signaling pathways.[4] We previously reported that clear cell carcinoma tends to have a low proliferation rate, which could contribute to its poor prognosis and resistance to chemotherapy.[5,6] We also showed that cyclin-dependent kinase (CDK) 2 activity reduced because high p27 expression may suppress proliferation of clear cell carcinoma, and we confirmed that up-regulation of CDK2 activity enhanced the cytotoxic effects induced by DNA-damaging agents, such as cisplatin.[7] Furthermore, phorbol 12-myristate 13-acetate abrogates the cisplatin-induced activation of cell cycle checkpoint kinase (Chk) 1 and Chk2 expression and resulted in apoptosis of cisplatin-resistant ovarian serous adenocarcinoma cells.[8] Therefore, cell cycle and its checkpoint pathways can be exploited to enhance the cytotoxic effects of chemotherapeutic agents in ovarian clear cell carcinoma.

Activation of cell cycle checkpoints by DNA damage leads to transient arrest in gap 1 (G_1), synthesis (S), and G_2/mitotic (M) phases, which allows time for DNA repair and promotes cell survival.[9,10] When the DNA repair is incomplete, the cells undergo apoptosis. Thus, inhibiting Chk proteins is thought to enhance response to the DNA-damaging effects of cytotoxic drugs and radiosensitivity by abrogating DNA damage-induced S and G_2 checkpoints and the cell cycle arrest in several types of cancer.[11–15] Recently, a novel ATP-competitive and selective Chk1 and Chk2 inhibitor, (*S*)-5-(3-fluorophenyl)-*N*-(piperidin-3-yl)-3-ureidothiophene-2-carboxamide (AZD7762) was developed and has entered clinical trials.[11,12] However, the effects of Chk inhibitors combined with the cytotoxic agents have not been evaluated in ovarian clear cell carcinoma. We, therefore, conducted the present study to determine whether AZD7762 enhanced the cytotoxic effects of cisplatin in ovarian clear cell carcinoma cells. We also explored the mechanisms of synergistic interactions between AZD7762 and cisplatin.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

The 4 human ovarian clear cell carcinoma cell lines used in this study were obtained as follows: RMG-I from Professor Shiro Nozawa, Keio University; KK from Dr. Yoshihiro Kikuchi, National Defense Medical College; and OVMANA from Dr. Hiroshi Minaguchi, Yokohama City University. TU-OC-1 was established by our department.[16] These cell lines were maintained in Dulbecco modified Eagle medium /Ham F-12 medium (Wako Pure Chemical Industries, Osaka, Japan) with 10% fetal bovine serum, 100-IU/mL penicillin, and 50-μg/mL streptomycin in a humidified atmosphere containing 5% carbon dioxide at 37°C.

Dose-Response Studies

The sensitivity of the cell lines to anticancer agents was determined by a cytotoxicity assay by using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan), according to the specifications of the manufacturer. Briefly, cells were incubated with various concentrations of the anticancer agents to obtain a dose-response curve for each agent. Concentrations for each drug were 10- to 1000-nmol/L AZD7762 (Axon Medchem BV, Groningen, The Netherlands), 1- to 30-μmol/L cisplatin (Sigma-Aldrich Co, St. Louis, MO), 1- to 1000-nmol/L paclitaxel (Sigma-Aldrich Co), and 1- to 1000-nmol/L 7-ethyl-10-hydroxycamptothecin (SN-38; Yakult Honsha Co, Tokyo, Japan), which is an active metabolite of camptothecin. After being incubated for 72 hours, 20 mL of Cell Counting Kit-8 solution was added to each well, and the plates were incubated for another 1 to 2 hours. Absorbance was measured at 450 nm with a microplate reader (iMark Microplate Absorbance Reader, Bio-Rad Laboratories, Inc, Richmond, CA).

Dose-Effect Analysis

AZD7762 was combined with each of the different anticancer agents at a fixed ratio that spanned the individual half maximal inhibitory concentration (IC_{50}) of each drug. The half maximal inhibitory concentration was determined based on the dose-effect curves by a cytotoxicity assay. Median effect plot analyses and calculated combination indices (CI) were analyzed by the method of Chou and Talalay.[17] CalcuSyn software (Biosoft, Ferguson, MO) was used to analyze data from the cytotoxicity assays in which cells were exposed to agents alone or combined with cisplatin and AZD7762. CalcuSyn provides a measure of the combined agents in an additive or synergistic manner. Chou and Talalay defined CI as synergistic ($CI < 0.9$), additive ($0.9 < CI < 1.1$), or antagonistic ($CI > 1.1$).

Western Blot Analyses

Cells were lysed in lysis buffer. A total of 50-μg protein was separated by electrophoresis on a 5% to 20% or 15% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The specific antibodies used were mouse anti-Chk1 (1:200 dilution, Santa Cruz Biotechnology, Inc, Santa Cruz, CA), rabbit anti–phospho-Chk1 (serine 296, 1:1000 dilution, Cell Signaling Technology, Beverly, MA), rabbit anti-Chk2 (1:200 dilution, Santa Cruz Biotechnology, Inc), rabbit anti–phospho-Chk2 (threonine 68, 1:1000 dilution, Cell Signaling Technology), mouse anti-CDC25A (1:200 dilution, Santa Cruz Biotechnology, Inc.), rabbit anti–phospho-Histone H2A.X (serine 139, 1:1,000 dilution, Cell Signaling Technology), rabbit anticleaved caspase-9 (1:500 dilution, Cell Signaling Technology), rabbit anticleaved PARP (1:1,000 dilution, Cell Signaling Technology), and mouse anti-actin (1:1,000 dilution, Sigma-Aldrich Co). These were visualized with secondary antimouse or antirabbit immunoglobulin G antibody coupled with horseradish peroxidase, using enhanced chemiluminescence (Amersham Biosciences, Bath, UK) according to the manufacturer's recommendation.

Immunofluorescence Studies

Cells were grown on Labtek chamber slides at 2000 cells per well and cultured with or without reagents (15-μmol/L cisplatin and/or 50-nmol/L AZD7762) for 24 hours. The cells were fixed in 1% paraformaldehyde for 15 minutes at 4°C, followed by incubation for 10 minutes with 0.2% Tween-20/phosphate-buffered saline (PBS). After blocking with 5% bovine serum albumin in 0.1% Tween-20/PBS for 1 hour at room temperature, cells were incubated with rabbit anti–phospho-Histone H2A.X antibody (serine 139, 1:150 dilution, Cell Signaling Technology) for 90 minutes at room temperature. The cells were incubated with antirabbit immunoglobulin antibodies conjugated with Alexa Flour 488 (1:1500 dilution, Molecular Probes, Carlsbad, CA) for 45 minutes at room temperature and stained with DAPI/PBS for 10 minutes at room temperature. The cells were mounted with Fluoromount (Diagnostic BioSystems, Pleasanton, CA) and visualized with a Keyence (Osaka, Japan) BZ-8100E fluorescence microscope.

Flow Cytometry

For analysis of cell cycle distribution, the cells (2×10^6) were trypsinized, collected by centrifugation, fixed in 70% ethanol at 4°C for 1 hour, and resuspended in PBS, containing 50-μg/mL propidium iodide and 0.1-mg/mL RNase. After 30 minutes at 37°C, the cells were analyzed with a FACSAria cytofluorometer (Becton Dickinson, Franklin Lakes, NJ).

Small Interfering RNA

Cells were seeded in 6-well culture plates at 2.5×10^5 per well (30%–50% confluence) in Dulbecco modified Eagle medium/F12 medium supplemented with 10% fetal bovine serum. The next day, cells were transfected with small interfering RNA (siRNA) against Chk1 (si-Chk1) (Cell Signaling Technology), Chk2 (si-Chk2) (Cell Signaling Technology), or control siRNA (Santa Cruz Biotechnology, Inc) to a final siRNA concentration of 100 nmol/L using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA).

Ovarian Clear Cell Carcinoma Xenograft Model

This study was carried out at the Laboratory Animal Research Center under the control of the Animal Research Committee, in accordance with the Guidelines for Animal Experimentation in the Faculty of Medicine, Tottori University, Yonago, Japan. RMG-I cells (5×10^6 viable cells in 0.25-mL PBS) were inoculated subcutaneously under aseptic conditions into the left flank of female nude mice. The mice were assigned randomly to one of 4 groups (10 mice per group), and treatment was started 10 days later as follows. Group 1, intraperitoneal (IP) PBS weekly; group 2, IP AZD7762 weekly (25 mg/kg per injection); group 3, IP cisplatin weekly (1.5 mg/kg per injection) for 4 weeks; and group 4, IP cisplatin with AZD7762 weekly for 4 weeks. Tumor size was measured with a caliper twice weekly, and tumor volume was calculated as: Tumor Volume (mm^3) = $\pi / 6 \times L \times W^2$, where L and W were the longer and shorter dimensions of the tumor, respectively.

Statistical Analyses

Analyses were performed with the JMP version 9 program (SAS Institute Inc, Cary, NC). Data are presented as means ± standard deviation. Means for all data were

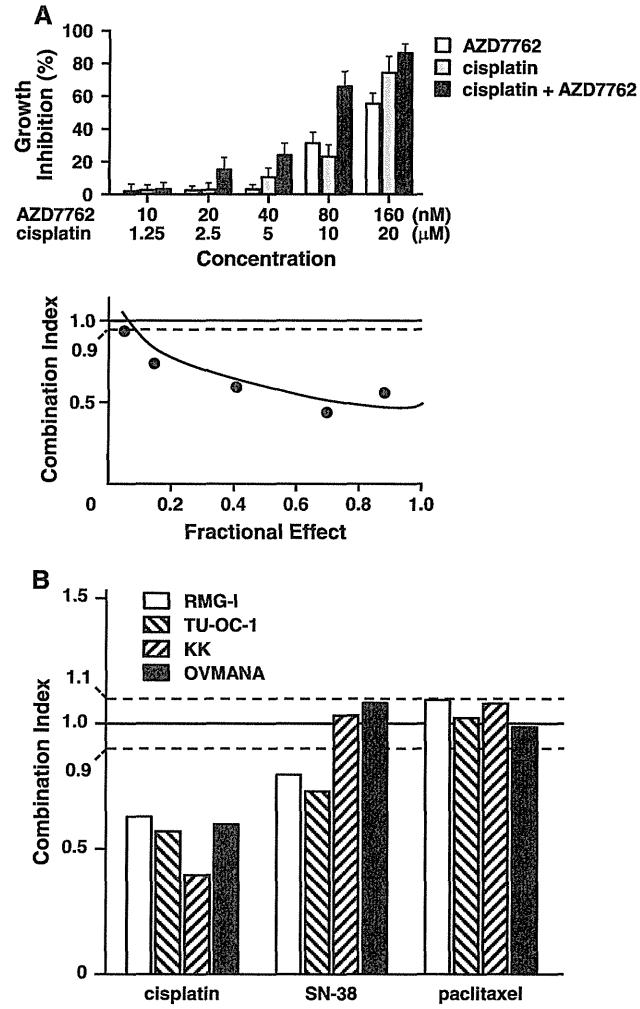


FIGURE 1. Effects of AZD7762 are synergistic with those of cisplatin. Cells were incubated with increasing concentrations of AZD7762 and cisplatin, 7-ethyl-10-hydroxycamptothecin (SN-38), or paclitaxel at a fixed ratio for 72 hours. A, Representative data from AZD7762 combined with cisplatin in RMG-I cells. Results are mean ± SD from 6 dishes. B, Data analyzed with CalcuSyn software to determine the CI. Chou and Talalay defined CI < 0.9, 0.9 < CI < 1.1, and CI > 1.1 as synergism, additivity, and antagonism of the 2 agents, respectively.

compared by one-way analysis of variance with post hoc testing. $P < 0.05$ was considered statistically significant.

RESULTS

Combination Effects of AZD7762 and Anticancer Agents

We analyzed the synergistic activity of combining AZD7762 with each anticancer agent from CI values calculated by the method of Chou and Talalay.[17] Data representative of AZD7762 combined with cisplatin in RMG-I cells are shown in Figure 1A. The CI value at an effective dose of 50 (effective dose means the percentage inhibition of cell growth using the drug combinations in the actual experiment) was less than 0.9 (synergism) for all 4 cell lines for cisplatin and 2 cell lines for SN-38 (Fig. 1B). However, the CI value was between 0.9 and 1.1 (additive) for all 4 cell lines for paclitaxel. Thus, when cisplatin was combined with AZD7762, synergistic effects were found in a greater number of cell lines.

AZD7762 Combined With Cisplatin Down-regulates Cell Cycle Checkpoints and Up-regulates the Apoptotic Pathway

We then examined whether the synergism arose from an increase in apoptosis induced by cisplatin. We confirmed that the protein expression levels of phosphorylated (p)-Chk1 at serine 296 and p-Chk2 at threonine 68 had increased and Cdc25A decreased after treatment with cisplatin alone in RMG-I and KK cells (Fig. 2A). AZD7762 inhibited

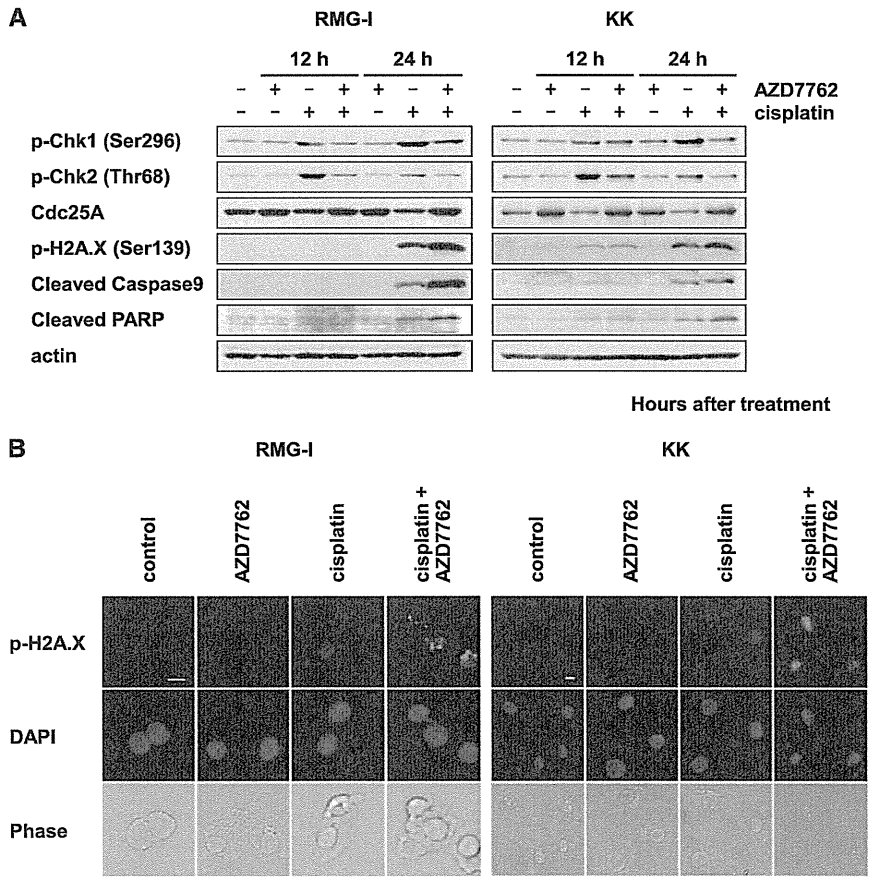


FIGURE 2. AZD7762 suppresses the cell cycle checkpoint pathways and enhances the apoptotic pathways induced by cisplatin in ovarian clear cell carcinoma cells. A, RMG-I and KK cells were treated at the indicated times with 15- or 7.5-μmol/L cisplatin and with PBS (control) and/or 50- or 150-nmol/L AZD7762, respectively. After being treated with cisplatin combined with AZD7762, the expression of p-Chk and p-Chk2 was suppressed and p-H2A.X, cleaved caspase 9, and cleaved PARP increased. The results shown represent duplicate experiments. B, RMG-I and KK cells were treated with AZD7762 and/or cisplatin for 24 hours and then fixed and immunostained for p-H2A.X. The nuclear expression of p-H2A.X increased dramatically after the treatment with AZD7762 and cisplatin in both cell lines. The results shown represent duplicate experiments. Scale bars, 10 μm.

phosphorylation of Chk1 and Chk2 effectively and stabilized Cdc25A in response to cisplatin. Interestingly, 24 hours after being treated with cisplatin and AZD7762, the protein expressions of p-H2A.X, cleaved caspase 9, and cleaved PARP were up-regulated. Immunofluorescence studies also showed that, compared to cisplatin alone, the increased formation of p-H2A.X protein in the nuclear foci was observed when cisplatin was combined with AZD7762 (Fig. 2B). Similar results were obtained in the other 2 cell lines (data not shown). These findings suggested that apoptosis induced with cisplatin in ovarian cancer cells may be enhanced by abrogating cell cycle check points, followed by up-regulation of unrepaired DNA damage by adding AZD7762.

AZD7762 Decreased S-Phase Fraction and Increased Cisplatin-Induced Cell Death

We assessed the cell cycle distribution by flow cytometry to confirm whether the combination treatment of cisplatin with AZD7762 influenced cell cycle distribution. After treatment with cisplatin alone, the proportion of RMG-I and KK cells in the S-phase fraction and the G_2/M phase were markedly increased (Figs. 3A, B). However, after treatment with cisplatin and AZD7762, the proportion of the cells in the G_0/G_1 and S phases decreased dramatically. Moreover, 48 hours after the combination treatment, the subG_1 population was significantly increased compared to treatment with cisplatin alone. Similar results were obtained in the other

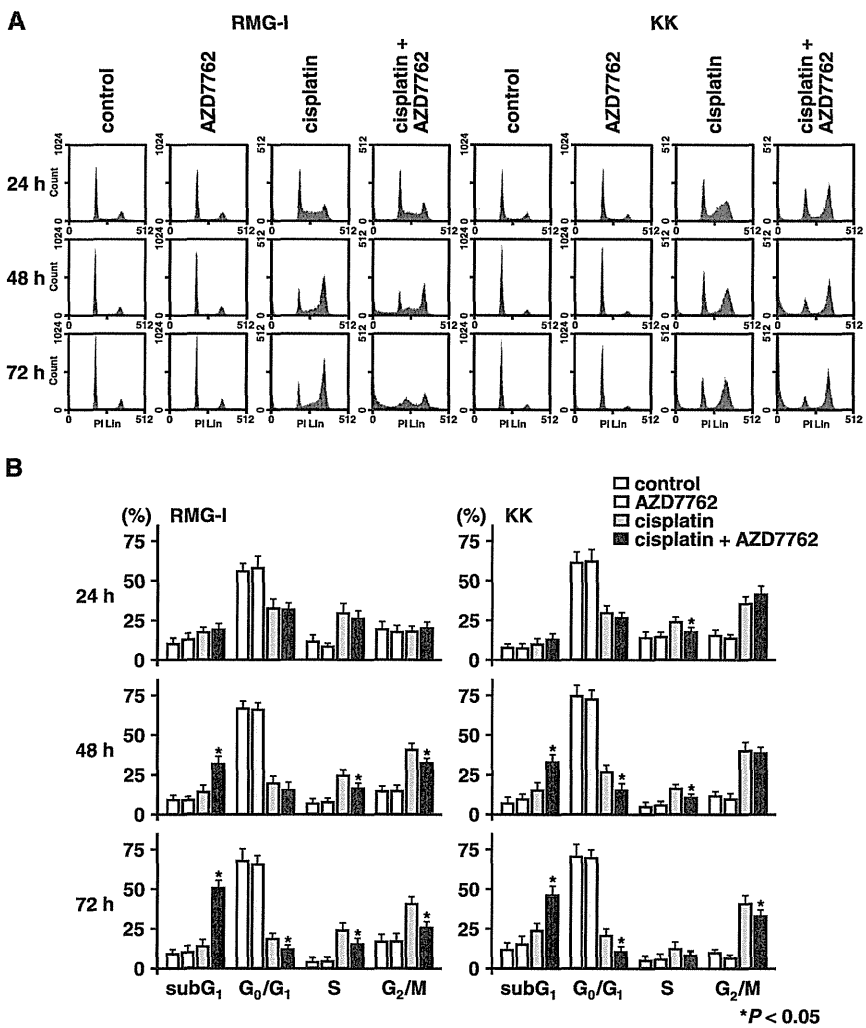


FIGURE 3. Effects of AZD7762 on the cell cycle distribution in response to cisplatin. Ovarian clear cell carcinoma cells RMG-I and KK were treated with PBS (control) or 5- or 3-μmol/L cisplatin and/or 50- or 150-nmol/L AZD7762, respectively. A, Representative data from the flow cytometry in RMG-I and KK cells are shown. Cell cycle distribution is displayed as propidium iodide (*x*-axis) versus cell number (*y*-axis). B, Cisplatin combined with AZD7762 decreased the S-phase fraction, and the cell cycle distribution was shifted to the subG1 phase for 48 and 72 hours in RMG-I and KK cells.

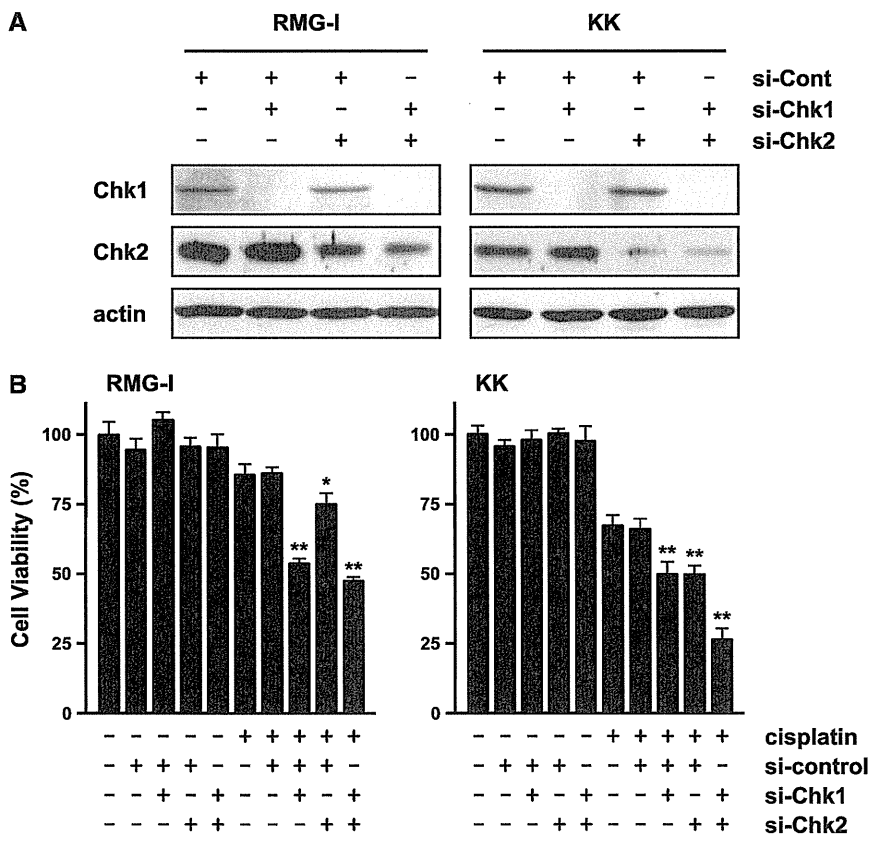
2 cell lines (data not shown). These results indicated that adding AZD7762 to cisplatin abrogated G_1- and S-phase arrest, after which the clear cell carcinoma cells died.

Cisplatin Sensitization in Clear Cell Carcinoma Cell Lines by Knockdown of Chk1 and Chk2

We next examined the relative contributions of inhibition of Chk1 or Chk2 by AZD7762 on sensitization of response to cisplatin in clear cell carcinoma cell lines by using siRNA to selectively knock down Chk1 and/or Chk2 in RMG-I and KK cells. After 24 hours of treatment with si-Chk1 or si-Chk2, expressions of Chk1 or Chk2 were down-regulated in RMG-I and KK cells, respectively (Figs. 4A, B). Sensitivity to cisplatin was increased upon treatment with si-Chk1 or si-Chk2 compared with nonspecific siRNA (si-control). Interestingly, simultaneous treatment with si-Chk1 and si-Chk2 dramatically increased sensitivity to cisplatin. Similar results were obtained in the other 2 cell lines (data not shown). These findings suggested that enhanced cisplatin sensitivity in clear cell carcinoma cells may be modulated by both Chk1 and Chk2 inhibition.

Cisplatin Combined With AZD7762 Reduced Tumor Growth in an Ovarian Clear Cell Carcinoma Xenograft Model

After confirming that AZD7762 enhanced cytotoxicity induced by cisplatin in vitro, we examined the effect of combined cisplatin and AZD7762 on the growth of subcutaneous tumors in an ovarian clear cell carcinoma xenograft. Female nude mice were given subcutaneous injections of RMG-I cells and then treated with PBS or cisplatin and/or



*** $P < 0.05$ vs. cisplatin + si-control, ** $P < 0.01$ vs. cisplatin + si-control**

FIGURE 4. Simultaneous inhibition of Chk1 and Chk2 expression by si-Chk1 and si-Chk2 increases cisplatin sensitivity in RMG-I and KK clear cell carcinoma cells. Cells were treated with 100 nmol/L si-Chk1 and/or si-Chk2 or a control siRNA (si-control) for 24 hours. A, si-Chk1 and si-Chk2 inhibited the expression of Chk1 and Chk2, respectively, in both RMG and KK cells. B, Cytotoxic effect of cisplatin was significantly enhanced by cisplatin combined with si-Chk1 and si-Chk2 in RMG-I and KK cells compared with other treatment conditions. Points represent mean ± SD from quadruplicate experiments.

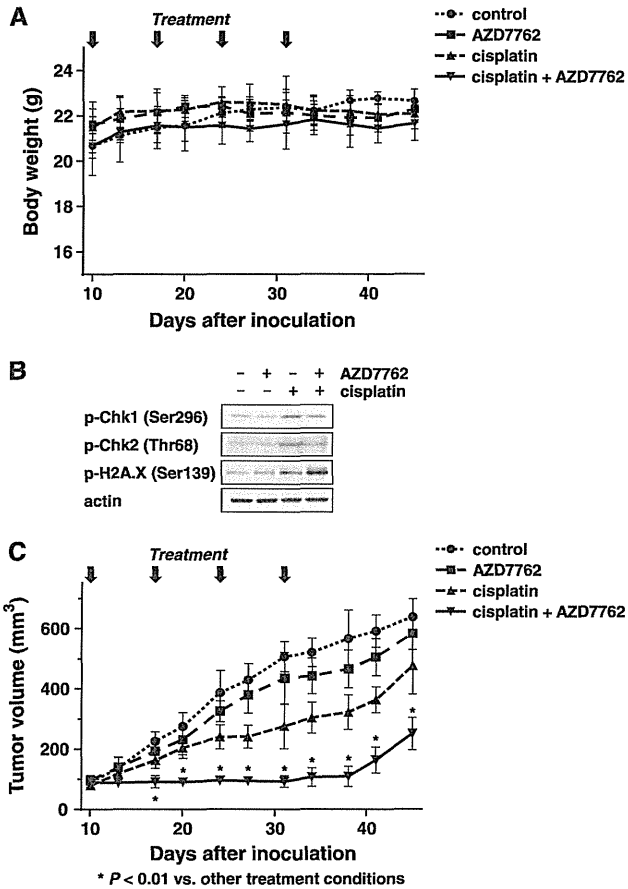


FIGURE 5. Treatments combining cisplatin and AZD7762 suppressed growth of subcutaneous tumors in mice with RMG-I cells implanted. A, Mean body weight of each treatment group. Error bars represent standard deviation. B, Levels of p-Chk1, p-Chk2, and p-H2A.X proteins were determined by Western blotting 24 hours after IP treatment with PBS (control), 25-mg/kg AZD7762, and/or 1.5-mg/kg cisplatin. The results shown represent duplicate experiments. C, In mice inoculated with RMG-I, the tumors were significantly smaller in the mice treated with AZD7762 combined with cisplatin compared with those under other treatment conditions ($P < 0.01$).

AZD7762. There were no signs of overt toxicity (weight loss or gross clinical signs) in any group (Fig. 5A).

To confirm that p-Chk1 and p-Chk2 were inhibited by AZD7762 in vivo, we performed Western blot analysis of tumor tissues (Fig. 5B). As expected, p-Chk1 and p-Chk2 proteins were up-regulated in tumors form the mice treated with cisplatin, and these were suppressed effectively in tumors from mice treated with both cisplatin and AZD7762. We also observed increased expression of p-H2A.X protein in tumors in mice treated with this combination.

In nude mice bearing RMG-I, the mean tumor volume of subcutaneous tumors in the group treated with AZD7762 combined with cisplatin was significantly smaller than that in the group treated with PBS, AZD7762, or cisplatin alone ($P < 0.01$; Fig. 5C). These findings indicated that combining cisplatin and AZD7762 suppressed tumor growth in the subcutaneous tumors of nude mice bearing RMG-I cells.

DISCUSSION

In this exploration of the combination effects of the Chk1 and Chk2 inhibitor AZD7762 with 3 cytotoxic agents used commonly to treat ovarian clear cell carcinoma, we found that AZD7762 and cisplatin had the strongest cytotoxic effects. We also showed that AZD7762 abrogated the G_1/S-phase cell cycle arrests induced by cisplatin and enhanced unrepaired damage to DNA. Thus, these findings suggest that inhibition of Chks up-regulates cisplatin-induced cytotoxicity in ovarian clear cell carcinoma cells.

Cisplatin-induced DNA damage triggers recruitment of multiprotein complexes and activates a number of pathways, including ataxia telangiectasia–mutated (ATM) and ATM and Rad3-related (ATR) signaling pathways.[9,10] Serine/threonine kinases of Chk1 and Chk2 are functionally redundant protein kinases that respond to checkpoint signals initiating ATM and ATR and play a critical role in determining cellular responses to DNA damage.[18] Checkpoint kinase 1 is mainly activated through phosphorylation mediated by ATR. Activated Chk1 phosphorylate Cdc25A leads to ubiquitin- and proteasome-dependent protein degradation and, downstream, to increased phosphorylation of CDK2. This limits its ability to drive progression from G_1 to S phase.[19] In contrast, Chk2 is activated mainly by ATM, and activated Chk2 phosphorylates Cdc25A.[18] Indeed, we confirmed that expression of p-Chk1 and p-Chk2 increased, whereas Cdc25A decreased after the cells were exposed to cisplatin. Furthermore, we observed that cells treated with cisplatin accumulated at S and G_2/M phases.

Preclinical studies have shown that AZD7762 potentiated the effects of DNA-damaging agents, such as cisplatin, gemcitabine, irinotecan, and paclitaxel, by abrogating drug-induced activation of Chk signaling pathways.[11–15] Similarly, we observed the synergistic effect of AZD7762 and cisplatin on inhibiting cell growth in clear cell carcinoma cell lines. AZD7762 also enhanced the cisplatin-induced up-regulation of p-H2A.X, reflecting a greater number of p-H2A.X molecules near sites of DNA damage, and activation of apoptotic pathways. These results suggested that AZD7762 enhanced the cytotoxicity induced by cisplatin such that this combination may be an effective treatment for ovarian clear cell carcinoma.

Although AZD7762 is an inhibitor of both Chk1 and Chk2, it has been suggested that Chk1 inhibition may play a central role in AZD7762-mediated chemosensitization.[20] Several studies have reported that knockdown of Chk1, but not Chk2, by siRNA produced sensitization to cisplatin and gemcitabine.[21,22] Additionally, the small molecule inhibitors of Chk1, PF-00477736, and PD-321852 enhanced the effects of cytotoxic agents.[20,23] In contrast, a novel small molecule inhibitor of Chk2, PV1019, had a synergistic effect

in combination with a topoisomerase I inhibitor in ovarian cancer cells.[24] We observed that simultaneous treatment with si-Chk1 and si-Chk2 dramatically increased sensitivity to cisplatin. Furthermore, recent studies have unveiled several roles for Chk1 in repairing damaged DNA (eg, homologous recombination), negatively regulated mitosis, stabilized stalled replication fork, and inhibited apoptosis.[25,26] Similar to Chk1, Chk2 also has several functions in controlling DNA repair, mitosis, and apoptosis.[27,28] Inhibiting these functions of Chk1 and Chk2, therefore, is thought to bring the potential to enhance the cell killing effects of DNA-damaging agents and radiotherapy. Thus, the chemosensitization effect of AZD7762 may be caused by not only inhibiting Chk1 but also Chk2 in clear cell carcinoma cells. Future studies may be needed to elucidate mechanisms for the synergistic interaction between Chk1/2 inhibitors and cytotoxic agents.

Finally, we confirmed the importance of cell cycle checkpoint pathways in cisplatin therapy in vivo in an ovarian clear cell carcinoma xenograft model. AZD7762 downregulated cisplatin induced activation of p-Chk1 and p-Chk2 expressions in the tumor, and combined AZD7762 and cisplatin suppressed growth of tumors in these mice compared with those treated with AZD7762 or cisplatin alone. The present study provides clear evidence that the downregulation of Chk pathways enhanced the effects of cisplatin in treatment both in vitro and in vivo.

In summary, our study showed that the Chk1/2 inhibitor AZD7762 enhanced the cytotoxicity of cisplatin in clear cell carcinoma cells. We also found that the synergistic interaction of AZD7762 and cisplatin may be related to abrogation of the cisplatin-induced G_1/S-phase cell cycle arrest that induces apoptosis. Furthermore, this combined treatment suppressed growth of tumors in nude mice injected with clear cell carcinoma cells. Therefore, we concluded that combining AZD7762 with cisplatin is worth exploring as a treatment for clear cell carcinoma. We hope that this combination therapy will improve the survival of patients with advanced ovarian clear cell carcinoma.

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ORIGINAL ARTICLE

Association between carotenoids and outcome of cervical intraepithelial neoplasia: a prospective cohort study

Takuma Fujii · Naoyoshi Takatsuka · Chisato Nagata · Koji Matsumoto · Akinori Oki · Reiko Furuta · Hiroo Maeda · Toshiharu Yasugi · Kei Kawana · Akira Mitsuhashi · Yasuo Hirai · Tsuyoshi Iwasaka · Nobuo Yaegashi · Yoh Watanabe · Yutaka Nagai · Tomoyuki Kitagawa · Hiroyuki Yoshikawa

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Abstract
Background It has been suggested that micronutrients such as alpha-tocopherol, retinol, lutein, cryptoxanthin, lycopene, and alpha- and beta-carotene may help in the prevention of cervical cancer. Our aim was to investigate whether serum concentrations and/or dietary intake of micronutrients influence the regression or progression of low-grade cervical abnormalities.
Methods In a prospective cohort study of 391 patients with cervical intraepithelial neoplasia (CIN) grade 1–2 lesions, we measured serum micronutrient concentrations in addition to a self-administered questionnaire about dietary intake. We evaluated the hazard ratio (HR) adjusted for CIN grade, human papillomavirus genotype, total energy intake and smoking status.

The Japan HPV and Cervical Cancer (JHACC) Study Group.

T. Fujii (✉)
Department of Obstetrics and Gynecology, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan
e-mail: fujiit@a5.keio.jp

N. Takatsuka · C. Nagata
Department of Epidemiology and Preventive Medicine, Gifu University, Graduate School of Medicine, Yanagito 1-1, Gifu, Gifu 501-1194, Japan

K. Matsumoto · A. Oki · H. Yoshikawa
Department of Obstetrics and Gynecology, Graduate School of Comprehensive Human Science, University of Tsukuba, 1-1, 1-chome, Tennoudai, Tsukuba, Ibaraki 305-8575, Japan

R. Furuta · T. Kitagawa
Department of Pathology, The Cancer Institute, Japanese Foundation for Cancer Research, 8-31, 3-chome, Ariake, Kouto-ku, Tokyo 135-8550, Japan

H. Maeda
Department of Transfusion Medicine and Cell Therapy, Saitama Medical Center, Saitama Medical University, 1981 Kamoda, Kawagoe, Saitama 350-8550, Japan

T. Yasugi · K. Kawana
Department of Obstetrics and Gynecology, The University of Tokyo, 3-1, 7-chome, Hongo, Bunkyo-ku, Tokyo 113-8655, Japan

A. Mitsuhashi
Department of Reproductive Medicine, Graduate School of Medicine, Chiba University, 8-1, 1-chome, Inohana, Chuo-ku, Chiba 260-8670, Japan

Y. Hirai
Department of Gynecology and Cytopathology, The Cancer Institute Hospital, Japanese Foundation of Cancer Research, 8-31, 3-chome, Ariake, Kouto-ku, Tokyo 135-8550, Japan

T. Iwasaka
Department of Obstetrics and Gynecology, Faculty of Medicine, Saga University, 1-1, 5-chome, Nabeshima, Saga, Saga 849-8501, Japan

N. Yaegashi
Department of Obstetrics and Gynecology, Tohoku University School of Medicine, 1-1 Seiryo-cho, Aoba-ku, Sendai, Miyagi 980-8574, Japan

Y. Watanabe
Department of Obstetrics and Gynecology, Kinki University, School of Medicine, 377-2 Ohnohigashi, Osakasayama, Osaka 589-8511, Japan

Y. Nagai
Department of Obstetrics and Gynecology, Faculty of Medicine, University of the Ryukyus, 207 Uehara, Nishihara-cho, Nakagamigun, Okinawa 903-0215, Japan

Results In non-smoking regression subjects, regression was significantly associated with serum levels of zeaxanthin/lutein (HR 1.25, 0.78–2.01, $p = 0.024$). This benefit was abolished in current smokers. Regression was inhibited by high serum levels of alpha-tocopherol in smokers ($p = 0.042$). In progression subjects, a significant protective effect against progression to CIN3 was observed in individuals with a medium level of serum beta-carotene [HR 0.28, 95 % confidence interval (CI) 0.11–0.71, $p = 0.007$), although any protective effect from a higher level of serum beta-carotene was weaker or abolished (HR 0.52, 95 % CI 0.24–1.13, $p = 0.098$). Increasing beta-carotene intake did not show a protective effect (HR 2.30, 95 % CI 0.97–5.42, $p = 0.058$).

Conclusions Measurements of serum levels of carotenoids suggest that regression is modulated by smoking status. Maintaining a medium serum level of beta-carotene has a protective effect for progression; however, carotene intake is not correlated with serum levels of carotenoids.

Keywords Human papillomavirus · Cervical intraepithelial neoplasia · Low-grade squamous intraepithelial lesion · Micronutrients · Carotenoids

Introduction

Persistent infection with human papillomavirus (HPV) may potentially lead to the development of cervical cancer. Most women are exposed to at least one type of genital HPV in their lifetime [1]. HPV infections often cause cervical intraepithelial neoplasia 1 (CIN1) [2]. Only a subset of individuals with CIN1 progress to CIN3 or invasive cervical cancer, suggesting that environmental cofactors are related to cervical carcinogenesis [3–5]. Numerous environmental candidates such as oral contraceptives, parity, smoking status, micronutrient status, nutrient intake, *Chlamydia trachomatis* infection and herpes simplex virus type 2 infection have been investigated as potential cofactors related to progression of CIN.

Much attention has been given to the role of dietary factors and serum micronutrients in the etiology of cervical cancer and CIN. Carotenoids and tocopherols are lipid-soluble micronutrients with potent antioxidant activities and modulatory effects on immunity. Recent publications have reported that the association of carotenoids and tocopherols with reduced risk has not been observed consistently [6–10]; however, these inconsistent results may be due to the study designs. Furthermore, the majority of case–control studies of the associations between micronutrients and outcome of CIN were conducted to assess either dietary intake or circulating micronutrients only [7–9, 11].

Foods are composites of several biologically active dietary components. Micronutrients in foods, as well as other possible anti-carcinogenic compounds such as detoxification enzymes, may have synergistic effects and interact with one another [11–13]. A recent multi-center cohort study reported an association between dietary intake of micronutrients and outcome of CIN. However, this study reported no information about circulating micronutrients [6]. Conversely, some prospective cohort studies reported an association between circulating micronutrient levels and outcome of CIN but no information about dietary intake [14, 15]. Both dietary intake and circulating serum concentrations of micronutrients are important in assessing the role of micronutrients in cervical carcinogenesis. We previously conducted a case–control study including 156 pairs of women with CIN1–3 and matched controls with normal cytology and found an inverse relationship between serum levels of alpha-carotene, lycopene and zeaxanthin/lutein and the risk of CIN development [16]. Because retrospective analysis of previous study findings provides only limited information, we report here the results of a prospective study that was conducted in an attempt to confirm these findings.

Materials and methods

Study design

We used follow-up data from the Japan HPV and Cervical Cancer Study, a prospective non-intervention cohort study conducted to identify determinants of low-grade squamous intraepithelial lesion (LSIL)/CIN regression and progression. Among a total of 570 study subjects with low-grade cervical abnormalities (cytological LSIL and histological CIN1/2) recruited from nine hospitals between 1998 and 2004, 391 women with data concerning serum micronutrients and complete entry questionnaires were enrolled in the present study. Details of the design, methods and primary results have been provided elsewhere [17, 18]. Participants entered the study only after voluntarily giving signed, informed consent. The subjects were routinely followed at 3- to 4-month intervals and received cytology and colposcopy examinations at each visit. To avoid interference of the biopsy procedure on the natural course of the disease, cervical biopsy was performed only when women had HSIL smears and major colposcopic changes that were suggestive of progression to CIN3 or worse. Progression was defined as histological CIN3 lesions or worse, diagnosed on central pathology review. We defined regression as at least two consecutive negative smears and normal colposcopy. Women were regarded as having persistent lesions when they did not have either regression or

progression over the period of follow-up. At enrollment, study subjects were tested for cervical HPV-DNA and circulating serum micronutrients. Information about smoking and dietary intake was obtained from a self-administered questionnaire. Participants were not obliged to answer the questionnaire and their participation was unrelated to their clinical evaluation, treatment or follow-up evaluation. The simplified diet history questionnaire used in the current study had been developed and validated previously [19]. Originally, a prototype diet history questionnaire including 169 traditional Japanese foods and dishes was developed. To alleviate the participants' burden, our simplified diet history questionnaire was developed to employ a stepwise regression method to select from the 169 diet history questionnaire items. This simplified questionnaire was composed of 14 categories: (1) dishes of meat and vegetables; (2) meat (without dishes including vegetables); (3) fish; (4) cereals; (5) eggs and soybean products; (6) vegetables; (7) seaweed; (8) juice; (9) fruits; (10) milk and dairy products; (11) desserts and snacks; (12) pickles; (13) seasoning; and (14) alcoholic beverages. Supplement use was not assessed in this study because of a lack of complete information regarding availability. Because it was impossible to distinguish between intake of alpha- and beta-carotene from the questionnaire, total carotene intake was described. Questions on smoking habits included status (never, former or current smoker) and intensity (number of cigarettes smoked per day).

Circulating micronutrients

Blood was collected in foil-wrapped glass tubes without heparin. Serum was separated by centrifugation at $1{,}000 \times g$ for 10 min and stored in the dark at −70 °C prior to sample preparation. Serum levels of retinol, alpha-tocopherol and various carotenoids were determined by a high-pressure liquid chromatography method described previously [21].

Statistical analysis

The association between smoking status and nutrient intake was analyzed by one-way analysis of variance. The association between smoking status and serum micronutrients was analyzed by analysis of covariance. The data were adjusted for age, body mass index (BMI) and alcohol intake frequency. For regression or progression, time to event was measured from the date of the index visit to the date of the visit at which cytological transition to normal or CIN3 was first detected. To estimate the association between the CIN outcomes and circulating serum micronutrients, serum micronutrient tertiles were examined. Hazard ratios (HRs) and 95 % confidence intervals (CIs) for each tertile with reference to the lowest tertile were calculated using a proportional hazard model. For nutrient intake, identical estimation was conducted. The Brinkman Index (BI) was calculated by multiplying the average number of cigarettes smoked per day by the smoking years. We detected HPV-DNA in exfoliated cervical cells by a PCR-based methodology described previously [20]. HPV DNA was amplified by PCR using consensus-primers (L1C1/L1C2 + L1C2M) for the HPV L1 region. HPV genotypes were identified by a restriction fragment-length polymorphism (RFLP) PCR method that has been shown to identify at least 26 genotypes of genital HPV [18]. HRs were adjusted for potential confounders, including CIN grade, HPV genotype, age, total energy intake and smoking status. Statistical analyses were performed using Stata statistical software, release 11.1 (Stata Corporation; College Station, TX, USA).

Results

Of the 570 women enrolled in the parent study, 391 met the eligibility requirements of the current study for tests of serum micronutrients and completion of entry questionnaires. Of these, 329 and 62 women were diagnosed as CIN1 and CIN2, respectively. The mean age of the women was 36.3 years (median 36.0, range 19–54). Of the 391 women, regression, persistence and progression occurred in 218, 135 and 38, respectively.

Influence of smoking status on circulating levels and intake of micronutrients

At enrollment, 190 women had never smoked, while 142 women were current smokers (BI >100). Data from three women were lost and the remaining 56 women were past smokers. We found a 22 and 10 % decrease in carotene and vitamin E intake in current smokers compared with non-smokers, respectively (Table 1). Among the three groups, there was a significant difference in the intake of fiber, calcium, carotenes, vitamin A, vitamin C and vitamin E. As shown in Table 2, current smokers had significantly lower serum levels of alpha-carotene, beta-carotene and cryptoxanthin compared with non-smokers. Smokers had marginally lower levels of lycopene. Retinol, zeaxanthin/lutein and alpha-tocopherol were not related to smoking status.

The effects of serum micronutrients and nutrient intake in regression subjects

Significantly more inhibition of regression was observed in women in the middle tertiles of serum alpha-tocopherol

Table 1 Relationship between estimated daily nutrient intake and tobacco smoking status

Nutrient intake per day	Non smokers ($N = 190$)		Past smokers ($N = 56$)		Current smokers ($N = 142$)		p value
	Mean	SD	Mean	SD	Mean	SD	
Total energy intake (kcal)	2,220.1	576.1	2,221.6	679.7	2,149.1	574.9	0.520
Protein intake (g)	85.2	26.2	85.2	31.0	79.4	27.3	0.127
Fat intake (g)	60.2	21.9	62.9	27.2	59.0	22.6	0.566
Carbohydrate intake (g)	329.5	78.3	325.2	85.6	315.2	74.6	0.255
Fiber intake (g)	5.3	1.9	5.2	2.0	4.6	1.8	0.004
Calcium intake (mg)	740.8	292.2	738.3	337.6	620.9	274.2	0.001
Retinol intake (μg)	284.6	219.1	302.4	176.9	331.2	624.7	0.597
Carotene intake (μg)	4,943.5	2,439.7	4,856.3	2,532.1	3,866.8	2,083.5	0.000
Vitamin A intake (IU)	3,430.6	1,587.5	3,424.3	1,546.9	2,954.2	2,197.4	0.049
Vitamin C intake (mg)	134.0	65.6	133.3	65.9	113.4	56.4	0.008
Vitamin D intake (IU)	76.4	48.8	69.3	40.6	66.9	53.7	0.213
Vitamin E intake (mg)	8.4	2.8	8.3	3.2	7.5	2.7	0.021
Salt intake (g)	13.5	4.1	13.7	4.8	12.8	4.5	0.291
Cholesterol intake (mg)	323.7	122.6	322.9	160.2	304.7	137.5	0.412

Analysis of variance was used to examine the differences in the mean values of factors among groups

SD standard deviation

Table 2 Relationship between serum micronutrients and tobacco smoking status

	Non-smoker ($N = 190$)		Past smoker ($N = 56$)		Current smoker ($N = 142$)		P value
	Adjusted mean	95 % CI	Adjusted mean	95 % CI	Adjusted mean	95 % CI	
Serum retinol (μg/dL)	59.23	56.42–62.04	59.70	54.59–64.81	60.88	57.24–64.51	0.695
Serum α-carotene (μg/dL)	9.70	8.58–10.82	7.47	5.43–9.51	7.23	5.78–8.68	0.003
Serum β-carotene (μg/dL)	58.05	50.77–65.33	46.61	33.36–59.85	41.02	31.60–50.44	0.003
Serum zeaxanthin/lutein (μg/dL)	54.93	50.77–59.09	54.06	46.50–61.62	49.88	44.50–55.26	0.205
Serum cryptoxanthin (μg/dL)	31.19	25.61–36.76	23.61	13.46–33.76	21.27	14.05–28.49	0.03
Serum lycopene (μg/dL)	30.00	26.76–33.22	34.68	28.80–40.55	27.23	23.04–31.41	0.06
Serum α-tocopherol (μg/dL)	881.68	817.51–945.84	953.15	836.40–1,069.91	873.56	790.50–956.63	0.414

Analysis of covariance was used to examine the differences in the mean concentrations of the serum levels of micronutrients that are related to the effect of the smoking status. The data were adjusted for age (20–29, 30–39, or 40–54 years), BMI and alcohol intake frequency (0, 1–6, 7/week)

(HR 0.68, 95 % CI 0.49–0.95) as compared with women in the lower tertiles, but the linear trend was not statistically significant ($p = 0.882$). From the questionnaire, high-load intake of retinol significantly inhibited the regression (adjusted model: HR 0.59, 95 % CI 0.40–0.89) but the linear trend was not significant (Table 3).

Because serum levels of most carotenoids were low and carotene intake was small in smokers, the regression group was sub-analyzed stratifying by smoking status (never or current smokers) as shown in Tables 4 and 5. In non-smokers (Table 4), regression was observed in women in the upper tertiles of serum zeaxanthin/lutein (HR 1.25, 95 % CI 0.78–2.01) as compared with women in the lower and middle tertiles, and the linear trend was statistically significant ($p = 0.024$). In current smokers, this was statistically abolished as shown in Table 5. In current smokers, a significant inhibition of regression was observed in women in the middle tertiles for serum alpha-tocopherol (HR 0.53, 95 % CI 0.27–0.94) as compared with women in the lower tertiles, and the linear trend was significant ($p = 0.042$) in the adjusted model (Table 5).

Effect of serum micronutrients and nutrient intake in progression subjects

In Table 6, a significant inverse relationship was observed in subjects with a medium level of serum beta-carotene (HR 0.28, 95 % CI 0.11–0.71, $p = 0.007$), although these

Table 3 HR of regression from entire CIN1/2 according to the serum micronutrients and nutrient intake questionnaire

	n	Person-months	Events	Cumulative 2-year rate (95 % CI)	Hazard ratio for regression (95 % CI)			
					Unadjusted	*p* value	Adjusted model	*p* value
Serum retinol							*p* for trend	0.812
Low (<55.2)	128	1,715.6	74	62.5 (53.6–71.4)	1		1	
Medium (55.2–67.9)	132	1,689.8	77	63.2 (54.4–72.0)	1.06 (0.77–1.46)	0.709	1.19 (0.86–1.65)	0.301
High (>67.9)	131	1,763.5	67	57.8 (48.6–67.4)	0.87 (0.62–1.21)	0.399	0.87 (0.62–1.22)	0.423
Serum α-carotene							*p* for trend	0.472
Low (<5.1)	127	1,654.9	71	60.9 (51.9–70.0)	1.00		1.00	
Medium (5.1–9.7)	133	1,750.0	68	57.3 (48.2–66.8)	0.91 (0.65–1.27)	0.574	1.00 (0.71–1.41)	0.984
High (>9.7)	131	1,764.0	79	65.2 (56.5–73.9)	1.04 (0.75–1.43)	0.828	1.26 (0.89–1.80)	0.19
Serum β-carotene							*p* for trend	0.095
Low (<28.3)	129	1,679.7	66	56.7 (47.7–66.2)	1.00		1.00	
Medium (28.3–57.6)	131	1,755.9	75	62.7 (53.8–71.6)	1.10 (0.79–1.53)	0.581	1.17 (0.83–1.66)	0.364
High (>57.6)	131	1,733.3	77	64.0 (55.2–72.9)	1.12 (0.80–1.56)	0.511	1.34 (0.93–1.93)	0.115
Serum zeaxanthin/lutein							*p* for trend	0.235
Low (<42.9)	130	1,645.9	76	62.7 (53.8–71.6)	1.00		1.00	
Medium (42.9–57.3)	130	1,803.1	70	58.1 (49.2–67.2)	0.85 (0.62–1.18)	0.341	0.97 (0.69–1.36)	0.868
High (>57.3)	131	1,719.9	72	63.5 (54.2–72.7)	0.89 (0.65–1.23)	0.488	1.05 (0.75–1.48)	0.768
Serum cryptoxanthin							*p* for trend	0.215
Low (<11.2)	129	1,659.5	74	63.9 (54.8–73.0)	1.00		1.00	
Medium (11.2–22.1)	130	1,754.7	67	56.8 (47.8–66.2)	0.87 (0.62–1.21)	0.406	0.91 (0.65–1.28)	0.592
High (>22.1)	132	1,754.7	77	63.1 (54.3–71.9)	0.99 (0.72–1.37)	0.974	1.07 (0.76–1.51)	0.694
Serum lycopene							*p* for trend	0.638
Low (<19.8)	129	1,713.7	69	58.6 (49.7–67.9)	1.00		1.00	
Medium (19.8–35.8)	131	1,780.3	79	66.3 (57.4–75.0)	1.07 (0.78–1.48)	0.67	1.07 (0.76–1.49)	0.705
High (>35.8)	131	1,674.9	70	58.5 (49.4–67.8)	1.02 (0.73–1.42)	0.914	1.08 (0.77–1.52)	0.662
Serum α-tocopherol							*p* for trend	0.882
Low (<753.0)	128	1,535.8	82	67.3 (58.7–75.6)	1.00		1.00	
Medium (753.0–983.9)	132	1,896.8	66	54.7 (45.9–64.0)	0.66 (0.48–0.91)	0.011	0.68 (0.49–0.95)	0.025
High (>983.9)	131	1,736.3	70	62.8 (53.2–72.3)	0.74 (0.54–1.01)	0.062	0.78 (0.56–1.09)	0.142
Retinol intake							*p* for trend	0.322
Low (<190.2)	130	1,555.8	74	62.8 (53.6–72.0)	1.00		1.00	
Medium (190.2–313.1)	130	1,755.6	74	63.3 (54.0–72.0)	0.89 (0.65–1.23)	0.484	0.76 (0.54–1.07)	0.12
High (>313.1)	131	1,857.5	70	57.8 (49.0–66.9)	0.80 (0.57–1.10)	0.172	0.59 (0.40–0.89)	0.011
Carotene intake							*p* for trend	0.325
Low (<3,281.4)	130	1,639.3	70	59.8 (50.6–69.1)	1.00		1.00	
Medium (3,281.4–5,042.8)	131	1,812.8	72	61.6 (52.5–64.7)	0.92 (0.66–1.28)	0.637	0.90 (0.63–1.28)	0.557
High (>5,042.8)	130	1,716.8	76	62.2 (53.5–71.0)	1.03 (0.74–1.42)	0.869	0.97 (0.65–1.46)	0.89
Vitamin A intake							*p* for trend	0.546
Low (<2,398.8)	130	1,601.8	70	61.5 (62.5–74.6)	1.00		1.00	
Medium (2,398.8–3,466.7)	131	1,834.7	72	59.7 (51.7–64.7)	0.90 (0.65–1.25)	0.541	0.91 (0.64–1.29)	0.599
High (>3,466.7)	130	1,732.4	76	62.6 (53.9–71.4)	1.01 (0.73–1.40)	0.948	0.93 (0.61–1.42)	0.727
Vitamin E intake							*p* for trend	0.147
Low (<6.7)	130	1,610.2	68	57.4 (48.3–66.7)	1.00		1.00	
Medium (6.7–8.7)	130	1,897.1	71	59.4 (50.5–68.5)	0.90 (0.64–1.25)	0.521	0.95 (0.66–1.39)	0.807
High (>8.7)	131	1,661.6	79	65.9 (57.1–74.6)	1.11 (0.80–1.54)	0.519	0.88 (0.54–1.43)	0.601

Cox's proportional hazard model showing the hazard ratio for regression in a cumulative 24-month period. The adjusted model was calculated by CIN grade (initial biopsy results; CIN1 or CIN2), HPV genotypes (HPV16/18/31/33/35/42/52/59, other high-risk types, low-risk types, or HPV negative) [17, 18], age, total calorie intake and smoking status (Brinkman index >100). The units of micronutrients are expressed as μg/dL